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Inhibitory Monoclonal Antibody against a (Myristylated) Small-Molecular-Weight Antigen from *Plasmodium falciparum* Associated with the Parasitophorous Vacuole Membrane

URSULA A. K. KARA,* DEBORAH J. STENZEL, LEANNE T. INGRAM, GILLIAN R. BUSHELL, JOSE A. LOPEZ, AND CHEV KIDSON

Queensland Institute of Medical Research, Bramston Terrace, Herston, Brisbane, Queensland 4006, Australia

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A small-molecular-weight antigen that occurs in asexual blood stages in synchronized cultures of *Plasmodium falciparum* was detected by a monoclonal antibody which inhibits parasite growth in vitro. This antigen, QF116, showed a molecular weight of 15,000 in parasite strain FCR-3K$^+$ from The Gambia and 19,000 in strain FCQ-27 from Papua New Guinea. The protein did not show significant glycosylation by galactose or glucosamine labeling but was found to be acylated by myristic acid. By using immunogold labeling and electron microscopy, the location of the antigen could be attributed to the parasitophorous vacuole membrane and to inclusions and vesicles residing within the cytoplasm of the erythrocyte host cell.

For most of its life cycle in humans, *Plasmodium falciparum* is located inside the host cell, with the exception of two stages during which it has a brief extracellular existence, the sporozoite and the free merozoite stages. A number of plasmodial antigens have been identified which appear to be associated with membranes of the parasite or the infected host cell. Considerable attention has been directed to the sporozoite surface membrane (18), the merozoite surface membrane (9, 12), and the plasma membrane (PM) of the parasitized erythrocyte (5). One of the intriguing events of the invasion process of the host erythrocyte by the merozoite is the formation of the parasitophorous vacuole. The membrane surrounding the vacuole is a simple membrane bilayer that is almost devoid of intramembranous particles at the early stage of infection (17). It has been suggested that components both of the erythrocyte membrane and the invading parasite (merozoite coat, rhoptry material, or both) make up the parasitophorous vacuole membrane (PVM) (25). In the course of schizogony, morphological changes occur in the vacuole membrane such that proteins presumably synthesized by the parasite become inserted therein. It has long been proposed that the parasitophorous vacuole contains antigenic material. Hope et al. (13) have suggested that antigen 5.1 is located within or close to the vacuole membrane, while it appears that S antigens accumulate within the vacuole (20, 22).

In this report we describe a small-molecular-weight antigen that is recognized by an inhibitory monoclonal antibody (MAb), which is associated with the PVM.

MATERIALS AND METHODS

Parasite cultures. Parasite strains FCQ-27 from Papua New Guinea (6) and FCR-3K$^+$ from The Gambia (as supplied by R. Howard) were grown in synchronous cultures as described previously (15). Parasite-infected erythrocytes at different stages of development were harvested on Percoll gradients (21).

MAb. The 8E7/55 hybridoma line used in this study was derived from mice immunized with schizonts of the D10 clone of FCQ-27 strain from Papua New Guinea, as described previously (4). MAb 8E7/55 is of the immunoglobulin G1 (IgG1) class, and for use in immunoelectron microscopic studies, it was purified from ascites fluid by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.).

Affinity purification of antigen. MAb 8E7/55 was covalently linked to cyanogen bromide-activated Sepharose (Pharmacia) according to the instructions of the manufacturer, and a 1% Triton X-114 extract of FCQ-27 was passed through the column. After extensive washing, the bound antigen was eluted with 0.2 M glycine (pH 2.8).

Inhibition of parasite growth in vitro. Affinity-purified antibody was added in various concentrations to quadruplicate cultures of synchronized FCQ-27 in 96-well plates. The wells contained 45 μl of RPMI 1640 medium–TES [*N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid]; 10% pooled human serum, with a 5% haematocrit and a 1 to 2% schizont parasitemia; and 5 μl of test antibody. Control wells were identical in all respects, except that the test antibody was replaced by either affinity-purified normal mouse IgG or medium alone. The cultures were incubated overnight at 37°C. Following merozoite release and subsequent invasion, 100 μl of medium was added containing 10% pooled human serum and supplemented with 30 μCi of [^{3}H]hypoxanthine (Amersham Radiochemical Centre) ml^{-1}. The parasites were allowed to develop for 40 h to the mature schizont stage and were harvested by pipetting well contents onto glass fiber disks (Whatman, Inc., Clifton, N. J.). These were washed in trichloroacetic acid-ethanol. The dry disks were immersed in 3 ml of toluene scintillant and counted in a scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

Immunofluorescence microscopy. The indirect fluorescence antibody test was performed on fixed and unfixed parasites. For fixed parasites, thin films of parasitized blood were fixed in methanol or acetone and incubated with MAb and fluorescein isothiocyanate-labeled, affinity-purified goat anti-mouse IgG as described elsewhere (24). The indirect fluorescence antibody test on unfixed parasites was performed by using 200 μl of parasite culture at a defined stage. Schizont-infected erythrocytes were treated with anti-erythrocyte antibody (Commonwealth Serum Laboratories, Mel-

* Corresponding author.

bourne, Australia) plus complement for 60 min at 37°C. A 2-h incubation of parasites with MAb at 37°C was followed by a 1-h incubation with fluorescein isothiocyanate-labeled secondary antibody at 37°C. Parasites were gently but extensively washed with medium after each incubation step. Parasites were mounted on microscope slides and examined by fluorescence microscopy.

Immunoelectron microscopy. Immunoelectron microscopy was carried out on thin-sectioned parasites of strains FCQ-27 and FCR-3K$^+$ at the ring, trophozoite, or schizont stage by using colloidal gold as an electron-dense marker. Samples of parasite culture were fixed with 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 10 min at room temperature. Cells were washed three times, dehydrated to 70% ethanol, infiltrated with resin (LR-White; London Resin Co. Ltd.), and polymerized at 50°C for 24 h. Thin sections were mounted on nickel grids and rinsed with water, nonspecific labeling was blocked with 5% bovine serum albumin in 20 mM Tris buffer, and thin sections were incubated with MAb for 60 min. One-hour incubations with secondary antibody (rabbit anti-mouse) and tertiary antibody (goat anti-rabbit; colloidal gold-conjugated; diameter, 10 or 15 nm; Janssen Pharmaceutica, Beerse, Belgium) followed. Sections were washed extensively after each incubation step. After sections were contrasted with uranyl acetate and lead citrate, they were viewed with an electron microscope (EM400; Philips). Control sections were labeled in parallel by using unrelated antibodies or by omitting the primary antibody.

Biosynthetic labeling studies. Malarial proteins were radiolabeled by uptake of 50 μCi of [2-^{3}H]glycine ml^{-1} or 25 μCi of [U-^{14}C]leucine ml^{-1} in RPMI 1640 medium. Glycoproteins were labeled by incorporation of 50 μCi of D-[6-^{3}H] glucosamine hydrochloride ml^{-1} or 25 μCi of D-[U-^{14}C] galactose ml^{-1}. All radiochemicals were purchased from Amersham Radiochemical Centre. Metabolic labeling with fatty acid was performed in RPMI 1640 medium by using 50 μCi of [9,10-^{3}H]myristic acid ml^{-1} coupled to bovine serum albumin. Synchronous cultures at the ring stage were labeled for up to 20 h, depending on the precursor that was used. Parasitized cells were washed and parasites were extracted by saponin treatment (23). Protease inhibitors (5 μg each of pepstatin, leupeptin, chymostatin, and antipain ml^{-1}) were added, and detergent-solubilized proteins were used for immunoprecipitation.

Immunoprecipitation. Portions of total parasite or Triton X-114 extracts from radiolabeled schizonts were incubated with ascites fluid of MAb overnight at 4°C. Immune complexes were then precipitated with rabbit anti-mouse IgG–protein A-Sepharose. The precipitate was washed in phosphate-buffered saline–Triton X-114 and the immune complex was eluted with sample buffer as described previously by Laemmli (14) for use in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10).

SDS-PAGE and autoradiography. Radiolabeled proteins were analyzed on SDS–polyacrylamide gels (12 or 15%) by the procedure of Laemmli (14). Following electrophoresis, gels were fluorographed with Amplify (Amersham Radiochemical Centre) for 30 min, dried, and autoradiographed on film (XAR 5; Eastman Kodak Co., Rochester, N.Y.) at −70°C.

Immunoblotting. After separation by SDS-PAGE, parasite proteins were electrophoretically transferred onto nitrocellulose paper and immunoreacted with MAb 8E7/55 and ^{125}I-labeled goat anti-mouse antibody, following the Western blot procedure of Towbin et al. (27).

RESULTS

Immunofluorescence with MAb 8E7/55. The reactivities of both acetone- and methanol-fixed ring-, trophozoite-, and schizont-infected erythrocytes of strain FCQ-27 from Papua

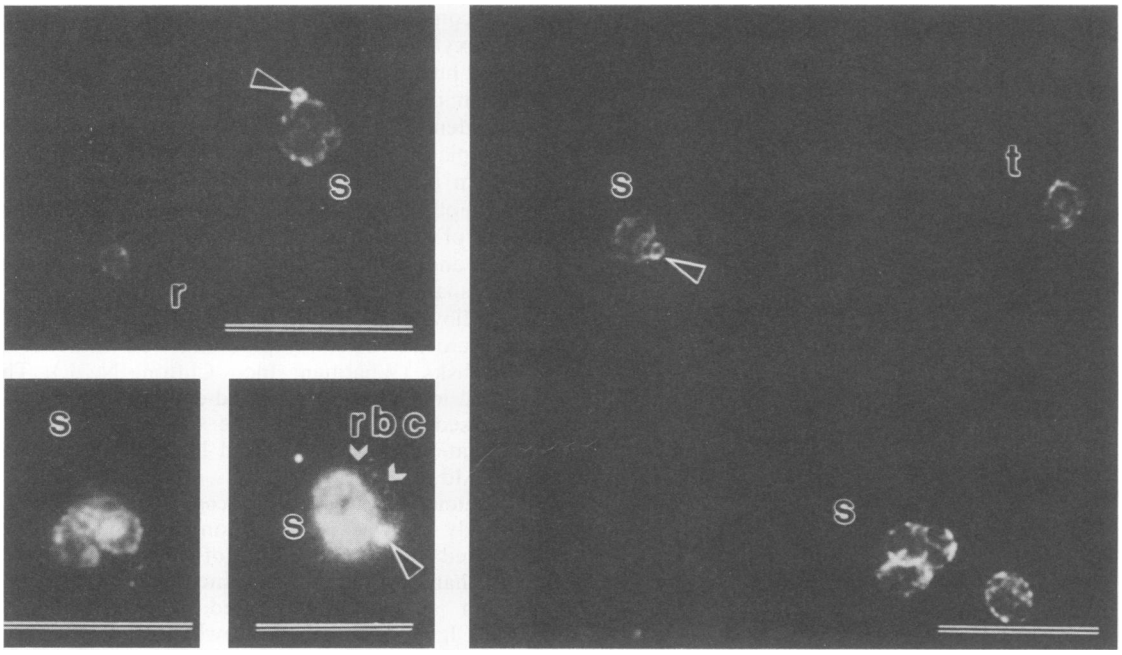

FIG. 1. Indirect immunofluorescence of fixed *P. falciparum* asexual blood stages reacted with MAb 8E7/55. Fluorescence micrograph shows ring (r)-, trophozoite (t)-, and schizont (s)-stage parasites in methanol-fixed smears of isolate FCQ-27. The antibody reacted with inclusions (open arrowheads) inside the host cell of schizont-infected erythrocytes (rbc). The erythrocyte membrane is marked by solid arrowheads. Bars, 10 μm.

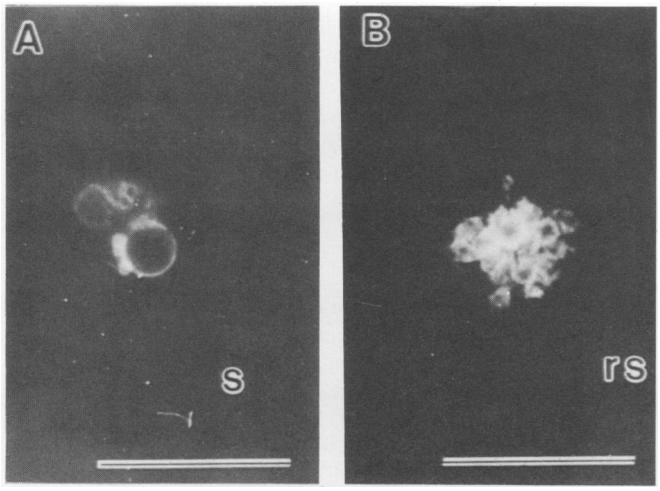

FIG. 2. Indirect immunofluorescence of unfixed *P. falciparum* asexual blood stages reacted with MAb 8E7/55 after the host erythrocyte was permeabilized with antibody and complement. Fluorescein staining is shown on schizont (s)-stage parasites and ruptured schizonts (rs) of isolate FCQ-27 (A) and FCR-3K$^+$ (B). Bars, 10 μm.

New Guinea and strain FCR-3K$^+$ from The Gambia with MAb 8E7/55 are illustrated in Fig. 1. Both isolates reacted strongly with the MAb at all stages, showing fluorescence around the parasite inside the infected erythrocyte. Ring-stage fluorescence, in general, was fainter than that at later stages. The antigen recognized by the antibody was also present in inclusions located in the cytoplasm of the host cell but outside the parasite. Similar results were obtained in the indirect fluorescence test with unfixed parasites (Fig. 2A), after they were permeabilized by treatment with anti-erythrocyte antibody and complement. Weak agglutination only was observed under these conditions. These parasites were still surrounded by a PVM (13a). The spherical appearance can be accounted for by the direct contact of the unfixed intact PVM with the external medium. After schizont rupture, clusters of membranous structures could be seen (Fig. 2B) which appeared to be associated with merozoites. Thus, fluorescence microscopic examination suggests that the antigen is located either in the parasitophorous vacuole (membrane) or in the PM of the parasite. This antigen was designated QF116.

Immunogold labeling studies. A more precise determination of the antigen site was obtained by electron microscopic examination. To discriminate between the location of the antigen in the parasitophorous vacuole, the PVM, or the PM, thin sections of parasite-infected erythocytes were subjected to immunogold labeling. When lightly fixed parasites were embedded in resin (LR White) without OsO_4 postfixation, all membranes appeared electron lucent and the vacuole cytoplasm displayed a granular density similar to that of the erythrocyte cytoplasm. The antigen was just detectable at the ring stage and was located around the parasite (Fig. 3A). In ring-stage parasites, the fixation conditions, which were sufficiently mild to allow subsequent immunogold labeling of antigen, resulted in a poor ultrastructural image of the PM and precluded precise localization of the antigen. In trophozoites the preservation of morphology was much better, but the close apposition of the PM and the PVM still made a definitive localization difficult (Fig. 3B). In mature schizonts (Fig. 4), however, in which the PM and the PVM were well separated, QF116 was clearly associated with the PVM and not with the PM or vacuole contents. Dense distribution of antigen was also seen associated with membrane-bound vesicles and inclusions residing in the cytoplasm of the host cell, as well as close to cytoplasmic clefts (Fig. 5B and C). Nonspecific gold labeling was very low on all sections.

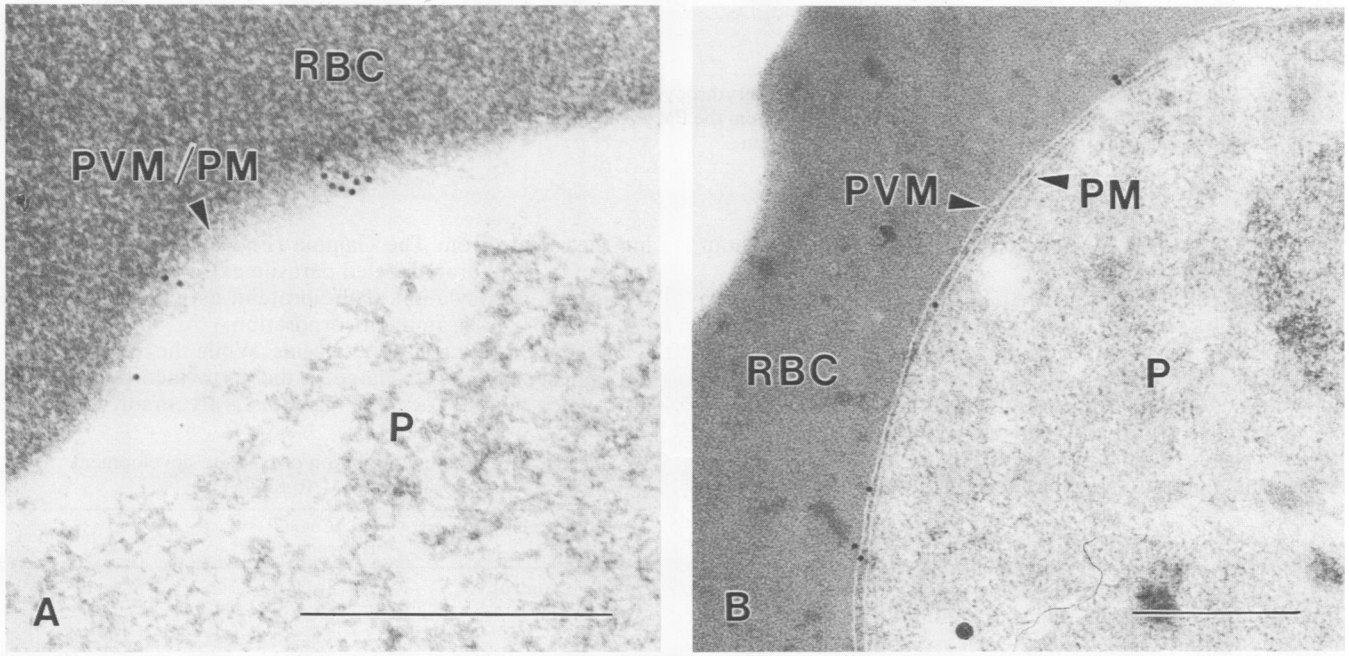

FIG. 3. Immunoelectron micrograph showing immature stage-infected erythrocytes from *P. falciparum* FCQ-27 labeled with Mab 8E7/55 and colloidal gold-IgG complex. (A) Ring-stage parasite surface labeled with gold particles (diameter, 15 nm). (B) Trophozoite-stage parasite labeled with gold particles (diameter, 10 nm). Bars, 0.5 μm. Erythrocyte (RBC) and parasite (P) are separated by both the PVM and the PM.

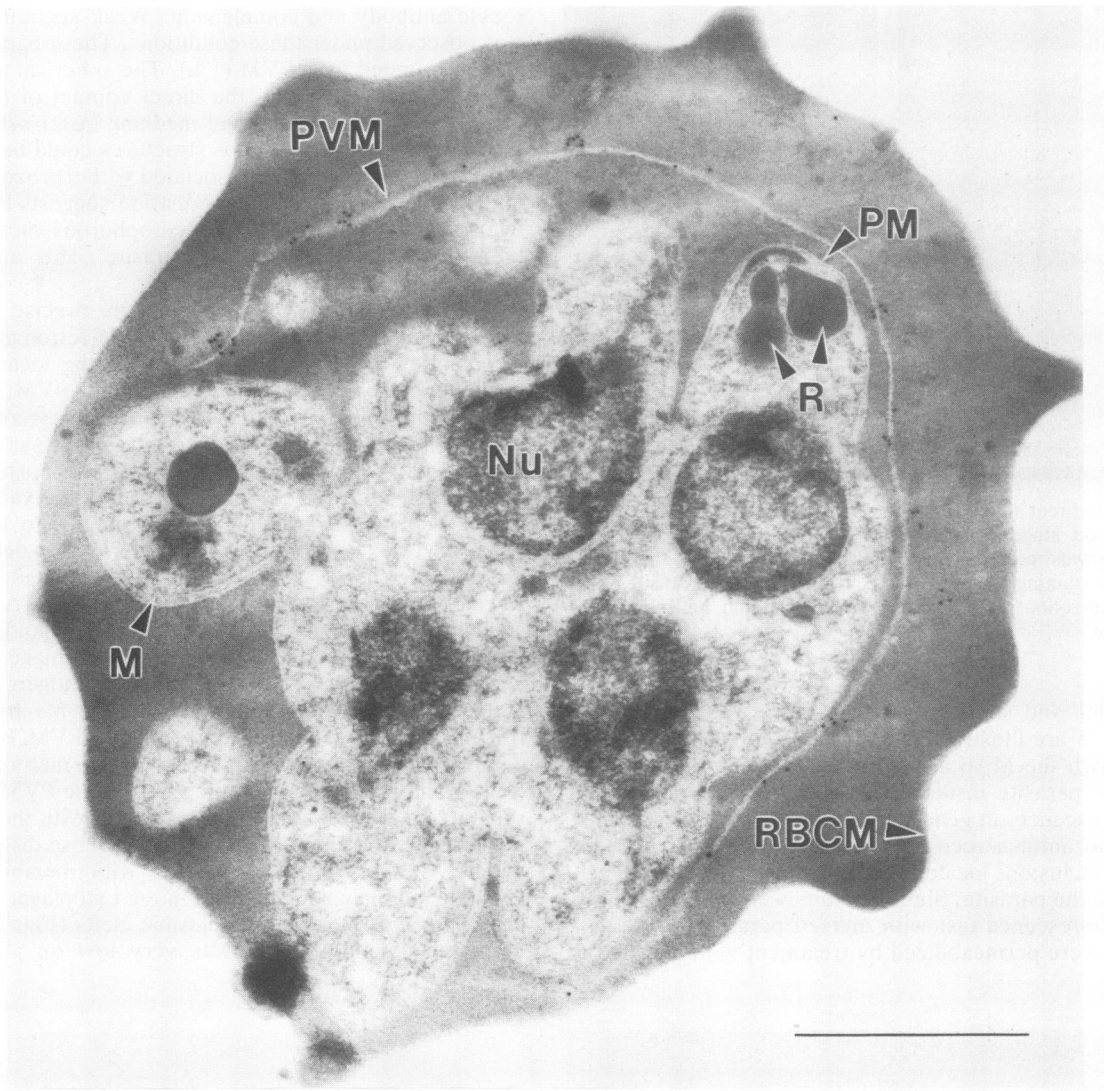

FIG. 4. Immunoelectron micrograph of schizont-infected erythrocyte (FCQ-27 strain) labeled with MAb 8E7/55 and colloidal gold-IgG complex. Label was seen on the PVM, which was separable from the PM at this stage. Abbreviations: Nu, nucleus; M, developing merozoite; R, rhoptry; RBCM, erythrocyte plasma membrane. Bar, 1 μm.

Labeling was absent on sections that were incubated without the primary antibody.

Inhibition assay. Purified MAb 8E7/55 inhibited the in vitro multiplication of FCQ-27 in a concentration-dependent manner, as determined by [^{3}H]hypoxanthine uptake (Table 1). Up to 80% inhibition was achieved during one cycle of parasite development at 0.6 mg of antibody ml^{-1}, when a normal mouse IgG fraction was used as a negative control. In comparison, 80% inhibition at 0.2 mg ml^{-1} was achieved in the same laboratory with a MAb against a rhoptry antigen (23).

Biosynthetic labeling with amino acids and carbohydrate. Isolate FCR-3K$^+$ from The Gambia was cultured in vitro with the two labeled amino acids glycine and leucine from the ring to the schizont stage. Schizont stage-infected cells were then harvested, parasites were isolated, and detergent-soluble material was immunoprecipitated with MAb 8E7/55. A single band with an M_r of 15,000 was detected by SDS-PAGE analysis of immunoprecipitated material by using the isolate from The Gambia (Fig. 6). Immunoprecipitates of carbohydrate-labeled parasite extracts did not show significant glycosylation of the protein, as judged by either galactose or glucosamine incorporation.

Metabolic labeling with fatty acids. While the molecule did not appear to be glycosylated, it did show incorporation of label when myristic acid was used as a precursor. Myristic

TABLE 1. Percent inhibition of parasite development caused by MAb 8E7/55

MAb 8E7/55 concn (μg ml^{-1})	Counts (10^3 cpm ± SD)[a]	% Inhibition
Negative control	80 ± 6	
25	63 ± 5	22
65	48 ± 5	40
420	25 ± 3	60
600	14 ± 3	82

[a] Values are means ± standard deviations of quadruplicate experiments.

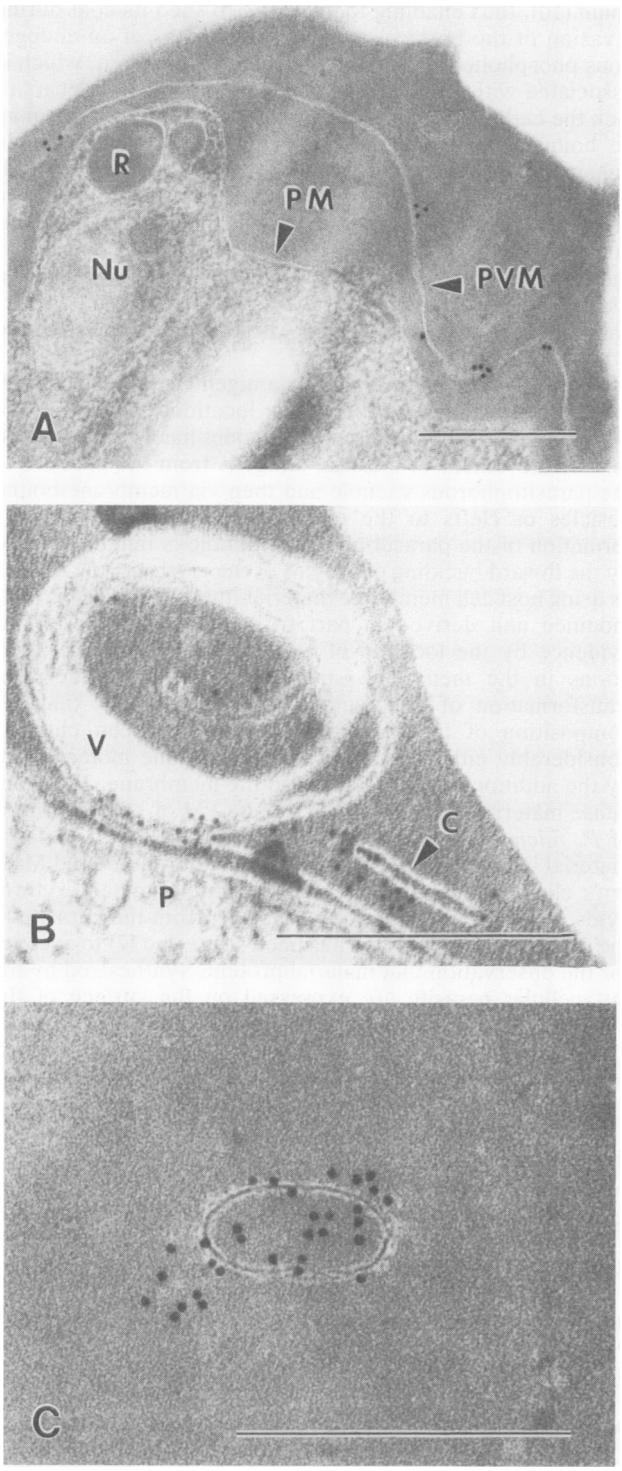

FIG. 5. Immunoelectron micrographs of *P. falciparum*-infected erythrocytes (FCQ-27 strain) labeled with MAb 8E7/55 and colloidal gold-IgG complex. (A) Schizont-infected erythrocyte showing a distinct separation of PVM from PM, with antibody labeling restricted to the vacuole membrane. Abbreviations: R, rhoptry; Nu, nucleus. (B) Membrane-bound cleft and multimembranous vesicle (V) showing antibody labeling. The parasite (P) is also shown. (C) Dense antibody labeling associated with a membrane-bound vesicle free of electron-dense material. Bars, 0.5 μm.

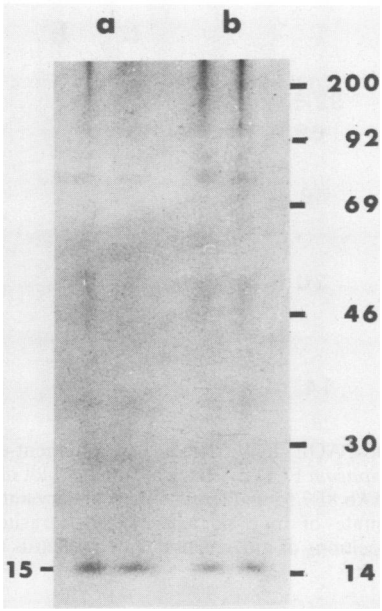

FIG. 6. SDS-PAGE analysis (12%) and fluorography of amino acid-labeled, detergent-extracted proteins from *P. falciparum* FCR-3K$^+$. Immunoprecipitation was with MAb 8E7/55 of parasites labeled with [^{14}C]leucine (lane a) and [^{3}H]glycine acid (lane b). Migration positions of molecular weight standards (in thousands) are indicated.

acid-labeled antigen was immunoprecipitated from the parasites FCR-3K$^+$ from The Gambia and FCQ-27 from Papua New Guinea with MAb 8E7/55, showing a band with a molecular weight of 15,000 and 19,000, respectively, on SDS-PAGE (Fig. 7, lane c, and 8, lane b, respectively).

Western blot transfer studies. After SDS-PAGE analysis of detergent-solubilized parasite extracts, proteins were electrophoretically blotted onto nitrocellulose paper. Immunodetection by incubation of total parasite extracts with the hybridoma culture supernatant of MAb 8E7/55, followed by incubation with iodinated goat anti-mouse antibody, gave

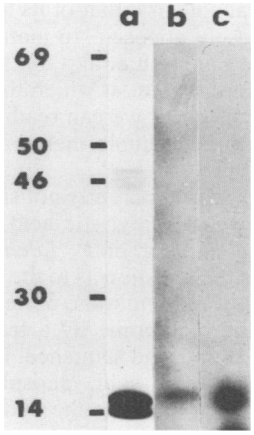

FIG. 7. SDS-PAGE analysis (12%) of detergent-extracted proteins from the *P. falciparum* FCR-3K$^+$. Lane a, Silver stain of affinity-purified vacuole antigen; lane b, immunoblot of purified antigen by use of MAb 8E7/55 and ^{125}I-labeled goat anti-mouse IgG; lane c, immunoprecipitate of myristic acid-labeled parasites with MAb 8E7/55. The positions of molecular weight standards (in thousands) are indicated.

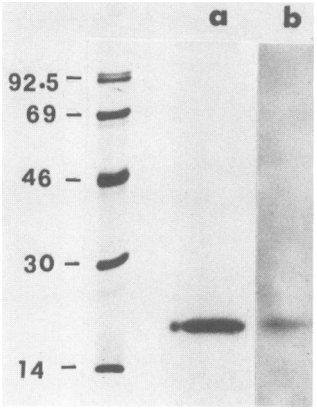

FIG. 8. SDS-PAGE analysis (15%) of detergent-extracted proteins of *P. falciparum* FCQ-27. Lane a, Immunoblot of total parasite extract with MAb 8E7/55 and iodinated secondary antibody; lane b, immunoprecipitate of myristic acid-labeled parasites with MAb 8E7/55. The positions of molecular weight standards (in thousands) are indicated.

bands with molecular weights of 15,000 and 19,000, depending on the parasite strains (Fig. 7 and 8). A similarly sized band was seen in silver-stained gels of SDS-PAGE-separated, affinity-purified antigen (Fig. 7, lane a). It remains to be determined whether the lower-molecular-weight band of the silver-stained doublet represents a degradation product of the 15,000-molecular-weight band.

DISCUSSION

The chemical characteristics of antigen QF116, which is recognized by an inhibitory MAb, indicate that it is a membrane-bound protein with a small apparent molecular weight. Its location can be attributed to the PVM of *P. falciparum*-infected erythrocytes, as well as clefts and vesicles within the cytoplasm of the host cell, as shown by immunofluorescence and immunoelectron microscopy. In erythrocytes infected by immature parasites, it has not been possible to localize the antigen more precisely than to the parasitophorous vacuole or either of its limiting membranes, since these are closely apposed. In mature schizonts, however, as shown by Langreth et al. (16), the parasitophorous vacuole enlarges to the point at which the PM and the PVM are well separated. Hence, we can readily demonstrate that labeling is only on the vacuole membrane, small vesicles, and clefts.

While the molecule is apparently not significantly glycosylated, it does incorporate myristic acid in its lipid moiety. The finding that a number of *P. falciparum* proteins are myristylated poses the question as to the purpose of this kind of protein modification. Membrane proteins most commonly are anchored in the membrane by a transmembrane-spanning hydrophobic amino acid sequence. Its replacement by a complex containing fatty acid, phosphatidylcholine, and carbohydrate has been found in the variant surface antigen of African trypanosomes (8). Biochemical results indicate that the parasite transferrin receptor of *P. falciparum*, too, is a glycoprotein that is acylated with myristic acid via 1,2-diacyl-*sn*-glycerol (11). The merozoite surface coat of *P. falciparum* may be constructed and function, in some respects, similarly to the trypanosome coat. The major merozoite surface glycoprotein, which contains both carbohydrate and myristic acid, may show a variant surface antigen-like type of linkage of the lipid tail to the peptide chain (10), thus enabling the parasite to shed its coat during invasion of the host cell, possibly by means of an endogenous phospholipase. In contrast, this PVM antigen, which is associated with a membrane inside the host cell, appears to lack the carbohydrate moiety; and its fatty acid residue may be bound to the peptide backbone in an ester or amide linkage, as has been described previously (19) for tumor virus proteins. Studies on the effect of lipases that are presently under way may give further insight into this question. Thus far, there has been no evidence as to whether the lipid moiety of the parasite antigen is immunogenic in itself, but a lipid complex interacting with membrane bilayer components could conceivably expose epitopes that would otherwise be cryptic.

Clues as to the function of this antigen can be obtained by the determination of its subcellular location. On the basis of electron microscopic evidence, a model has been proposed for the movement of malarial antigens from the parasite to the parasitophorous vacuole and then via membrane-bound vesicles or clefts to the erythrocyte membrane (2). The formation of the parasitophorous vacuole is initially caused by the inward buckling of the PM of the erythrocyte. As well as using host cell membrane material, the PVM appears to be modified and derived in part from the parasite itself, as evidence by the location of antigen QF116. Drastic alterations in the membrane structure are seen in the later transformation of the vacuole (1). This implies that the composition of the membrane must have been changed considerably either by removal of membrane molecules or by the addition of new material to the membrane. Electron-dense material appears at the parasite PM of several strains of *P. falciparum* cultured in vitro (3). This electron-dense material is also seen associated with membrane-bound Maurer's clefts in knob-positive, *Plasmodium*-infected erythrocytes. A route of intracellular transport from the parasite to the erythrocyte membrane has been suggested (7) to account for the observation that malarial proteins synthesized by the intracellular parasite are expressed on the surface of the infected cells. While the normal erythrocyte lacks the machinery for protein transport, which is found in less specialized cells, the parasite is able to export its own proteins. The parasite may well make special proteins for this purpose. They would be first exported through the parasite surface to the parasitophorous vacuole, and there they would interact with the membrane, possibly via a lipid tail. Such proteins could conceivably confer on the vacuole membrane the ability to form vesicles, which could provide the means to move other parasite proteins through the host cytoplasm.

The small-molecular-weight antigen we have described here is closely associated with both the PVM and vesicles, as well as cytoplasmic clefts, and it could conceivably be involved in the traffic of proteins. A similar role has been proposed by Simmons et al. (26) for antigen 5.1. Evaluation of the relationships, if any, between antigen 5.1 and antigen QF116 described here requires sequence data. The basis for inhibition of merozoite invasion by MAb 8E7/55 also remains to be established. Preliminary results obtained from electron microscopic studies (13a) suggest that PVM fragments containing antigen QF116 may cap free merozoites. Alternatively, it is feasible that a shuttling of membrane-bound antigen takes place from the PVM to the surface of the infected erythocyte, where it can interact with antibody, resulting in the blockage of this transport mechanism or the uptake of vital nutrients. A clarification of the mechanism of

inhibition is presently under way, as is an examination of the potential of these types of antigens as vaccine candidates.

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